RESEARCH PLAN PROPOSAL

Bioremediation of toxic metals from engineering industries by Arbuscular Mycorrhizal Fungi

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Introduction

The ill-treatment to nature has befallen to be the foremost reason for pollution. The unrestrained escalation of industrial activities and urbanization triggered a curse for the planet Earth leading to ecological imbalances, contamination of air, soil and water, risking flora and fauna and ultimately staking our own lives into an ambiguous game of contaminants and containment. Thus bioremediation can act as a game changer to battle against coarse industrial effluents churned out by the vigorous production of industries. The bioremediation is a noble technique for mitigation of organic pollutants which are not biodegradable on its own and is executed by employing microorganisms like bacteria and fungi (EPA 2012). The basic characterization involves *in-situ* and *ex-situ* bioremediation where the former calls treatment on the site and latter away from the site which can be anywhere (EPA 2012). Thus, it has come into lime light as an elite method for environment cleaning and surpassing all other erstwhile methods. Soil pollution by heavy metals has become the premier concern of talk of the entire environment crisis today. Usually Copper, Nickel, Zinc, Manganese and Iron present in trace amounts as natural constituents are harmless but over limiting the percentage have toxic effects on plants and humans as it get accumulated in food chain (Panda and Choudhary, 2005). Among all Cadmium, Arsenic, Lead, Chromium and Mercury are chief pollutants worldwide (Bempah, 2011).

The use of Arbuscular mycorhizzal fungi is a boon for the scientists as not only it causes sequestration of heavy metals but also enhance the nutrient content in soil (Barea *et al*., 1995). AMF are essential components of soil diversity of microorganisms because of increased yields, crops quality, flowering, fruiting, increased chances of survival of seedlings, reduced percentage of disease occurrence, more tolerance to drought, salinity, temperature etc., increased use of NPK fertilizers and reduction in soil erosion (Kumar, 1995).

Phytoremediation involves the treatment of polluted natural resource through the use of plants that mitigate the problem without the need to excavate the contaminant material and dispose of it elsewhere (Kumar, 1995). Certain plants called hyperaccumulators are able to bioaccumulate, degrade, or render harmless contaminants found in natural resources such as soils, water, and air. Bioaccumulation of Cs with was observed by Lasat *et al*., (1998). Phytostabilization entails the reduction of the mobility of substances in the environment by limiting the leaching of substances from the soil and sequestering pollutants in soil near the roots but not in plant tissues (Alkorta *et*

al., 2010). Another method is Phytoextraction which employs metal hyperaccumulator plant species to transport high quantities of metals from soils into the harvestable parts of roots and shoots (Chaney *et al*., 1997). Plants also reduce toxicity and sequester the xenobiotics through phytotransformation. The Trinitrotoluene phytotransformation has been extensively researched and a transformation pathway has been proposed (Subramanian *et al*., 2006).

The one of the most emerging technique is Mycoremediation which involves the removal or degradation of toxicants using fungi which acts as a suitable because of its tolerance to extreme physico-chemical conditions (acidity, pH, temperature, stress and aeration), they play a key role in ecosystem equilibrium, economical constraints are less so good for socio-economic purpose, most important their symbiotic association with different plants and ultimate contribution in increasing the nutrient content in bio-geochemical cycles. Successful treatments have been carried on Industrial Wastewater sludge (Zeyayllah, 2009), Petroleum hydrocarbons (Khalil, 2012), Dyes (Molla *et al*., 2012), paper and pulps effluents (Yunus, 2011). Mushrooms have also proven as effective machinery for Mycoremediation of waste and other pollutants. Luz *et al*., (2013) showed degradation of biodegradable plastic by *Pleurotus ostreatus*. Degradation of dyes using radioactive cellulosic-based waste simulates by mushroom by Rajput *et al*., (2011).

Review of Literature

Bioremediation: As a potential tool in amelioration of environmental pollution

Bioremediation technology has emerged as a potent tool because of its ability to remove or reduce degradation of pollutants but also for erasing the unwanted substances from soil, air and water from industries effluents (Cairney, 1993). Since bioremediation seems to be a good alternative to conventional clean-up technologies; substantial research in this field is rapidly increasing. (King *et al*., 1997). Algae and plants are very good at absorbing Nitrogen, Phosphorus, Sulfur, minerals and metals from the environment. Many algae and bacteria produce secretions that attract metals that are toxic in high levels. The metals are in effect removed from the food chain by being bound to the secretions (Caplan, 1993).

Environmental factors like nutrients i.e. supply of Carbon, Nitrogen and Phosphorus to already present microbes stimulate the production of enzymes for conversion of toxic compounds to less toxic compounds by the process of biostimulation (Vidali, 2001). The microbial growth depends upon pH, temperature and moisture. The parameters should be optimum but changes like increased pH of soil, may cause cell to shrink or increased temperature even causes cell death (Pritchard *et al*., 1992). Studies have shown that waste water from steel industry generates Total suspended solids (up to 4,000 mg/l, 1030kg/t), Lead (8 mg/l), Chromium (5 mg/l), Cadmium (0.4mg/l), Zinc (14 mg/l), Fluoride (20 mg/l). So treatment is necessary as it causes atmospheric pollution, discoloring, turbidity, dissolved oxygen depletion, algal and fungal growth. (Clescerl *et al*., 1999).

Phycoremediation has proven to be an effective and economic process of polluted waters. For example, the three microalgae employed were found to be efficient *Chlorella minutissima, Scendesmus species* & BGA example*, Nostoc* growed well in polluted habitats and showed gradual decrease in BOD, COD, and TDS and acted as antileachates for Nitrogen compounds and also used for algal manure (Sharma *et al*., 2013). White rot fungi *Coriolopsis rigida* strain LPSC no 232 has shown to degrade lignin, dyes, PAH, aliphatic crude oil fractions but along with association of AMF; have shown immense potential in improved plant growth and accumulation of nutrients (Saparrat, 2014). Phytoextraction of Chromium from tannery effluent contaminated soil by *Crotalaria juncea* and infestation with *Psuedomonas fluoroscens* greatly

increased chlorophyll content by 92% percent, biomass improvement by 99% as compared to uninoculated *C. juncea* plants. Thus, bioremediation is a potential technique for environmental cleanup (Agarwaal *et al*., 2014).

Removal of heavy metals by employing Arbuscular Mycorhizzal fungi

The mycorrhizal fungi are a symbiotic association between a vascular plant roots and fungi. They are commonly divided into Ectomycorrhizas and Endomycorrhizas.The former hyphae do not penetrate cells in the root and in latter the hyphae penetrate cell wall and invaginates the cell membrane (Harley *et al*., 1983). Endomycorrhizal fungi include Arbuscular, Ericoid, Orchid and Arbutoid as Ectoendomycorrhizas. Monotropoid includes a special category. Ericoid mycorrhizas have noticeable saprophytic characteristics which helps plants to receive nutrients from unrecompensed matter (Smith and Read, 1997). Arbutoid Endomycorrhizal fungi found in plant order *Ericales* and genera *Arctostaphylos* and *Arbutus*. The fungal hyphae form a Hartig net into outer cortical cells and allow transfer of nutrients from the fungi to plants and vice-versa. Monotropoid mycorrhizal fungi are a non-mutualistic parasitic interaction in which plant order *Ericaceae* and plants are hetrotrophic and mixotrophic to obtain Carbon from fungi partner. In orchids, the hyphae penetrate cells of embryo form hyphal coils within cells and obtain Phosphorus and Nitrogen (Moore, 2013).

Arbuscular Mycorrhizal fungi are soil microorganisms that establish mutual symbiosis with the majority of higher plants, providing a direct physical link between soil and plant roots (Barea *et al*., 1995). About 95% of the world's plant species belong to characteristically mycorrhizal families (Smith and Read, 1997) and potentially benefit from AM fungus-mediated mineral nutrition due to the fundamental role in biogeochemical element cycling (Jeffries, 1997). AM fungi can contribute to plant growth, particularly in disturbed or heavy metal contaminated sites, by increasing plant access to relatively immobile minerals (Barea *et al*., 2003), improves soil texture by binding soil particles into stable aggregates that resist wind and water erosion (Rillig *et al*., 2002) and by binding heavy metals into roots that restricts their translocation into shoot tissues (Dehn *et al*., 1987). The Fungi also accelerates the revegetation of severely degraded lands such as coal mines and waste sites containing high levels of heavy metals (Marx *et al*., 1979). The use of AMF for Phytoremediation process in biochemical and molecular aspects has been also investigated. (Biondi *et al*., 2014).

The heavy metal Cadmium tolerance to *Glomus species* and *Gigaspora species* with *Hordeum vulgare* in Cd polluted and unpolluted soils was observed by Tullio *et al*., (2003). In parallel to the above work Cd obliteration was seen in roots of *Trifolium subterraneum* using hyphae of *Glomus mosseae* (Joner and Leyval, 1997), *G. mosseae* and *Allium porrum* (Weissenhorn *et al*., 1993). Nickel was accumulated in *Berkheya codii* with *Gigaspora species* and *Glomus tenue* from ultramific soils of South Africa (Turnau *et al*., 2003).

Species of AMF like *Glomus constrictum*, *G. ambisporum, G. scutellospora and G. dipurpurescens* in interaction with *Andropogun geradii* had noticeable fertilizer amendment and Zinc tolerance to plant (Weissenhorn *et al*., 1994). Also, reduction of Zinc toxicity in grass *Festia rubra* by inoculation with *G. fasiculatum* in Zn contaminated soil was observed (Dueck *et al*., 1986*). Zea mays* and *A. capillaries* are potent hyperaccumulators and addition of *G. intradices* had demonstrated Lead uptake and also reduced Cd and Cu levels. (Liao *et al*., 2003). Diaz *et al*., (1996) studied influence of Zn, Pb capture on *Lygeum spartum* after association with *G. mosseae a*nd *G. macrocarpum*. Later Joner *et al* ., (2000) investigation lead to expression of metal binding capacity of *G. mosseae* with *T.subterraneum* and noticed binding capacity for Zn and Cd. Also AMF contribution in Ni and Cd in maize and bean plants was observed (Guo *et al*., 1996). Also sewage sludge effluents cause contamination in soils but can be resolved when harmful metals are removed with AMF inoculums (Val, 1999). *Solanum nigrum* grow well in polluted soils and the response was seen when inoculated with *Glomus mosseae*, *Glomus claodeum* and *G. intradices*. The result interpreted stem tissues to translocate Zn in higher proportion as compared to leaves but no significant effect in the plant translocation capacities (Paula, 2006). Ravimycin *et al*., (2012) observe effects on tannery effluents by using AMF on *Arachis hypogaea L.* About 5% concentration increase in parameters was seen as compared to raw effluents irrigation in comparison to AMF irrigated soils.

The appraisal in performance of contaminated soil can be more upgraded by inoculation of crops such as wheat when AMF was added (Khan, 2014). Mercury stress resistance was observed in *Nauclea orientalis* seedlings inoculated with AMF in roots in heightened amounts rather in shoots (Hanna, 2014). Sequestration of Cd in *Zea mays L.* under effects of *G. intradices, G. constrictum*, *and G. mosseae* in roots was clearly perceived by Lingzli (2014).

Materials and Methodology

1. Survey and soil sampling

The objective of soil sampling is to determine the percentage of nutrient and contamination accessible in soil, its key structure, and examine physical and biological properties to appraise the fertility, nature of soil and to quantify heavy metal accumulation, minerals and trace minerals. A sampling process considers the following process; taking the best representatives of samples, Analyzing the sample, interpretation of the analyzed results and giving future recommendations. Factors taken into account for a good soil sampling are as follows sampling time, Tools used in sampling, sampling depth, sampling frequency and tactics used in sampling (Mahler, 1994). There are many different methods of soil sampling but most preferred are random sampling and composite method which are as follows:

Random Sampling-This sampling can be done on entire field when field is uniform. It is most appropriate when discrepancy is less. This is done to observe enduring drifts, movement of nutrients, wastes and quantify them. In the present scenario, observation is also done using Global positioning system (Mason, 1992). A recent inference is from textile industry dye effluents for Cd, Pb and Zn estimation by the random sampling method (Basha *et al.*, 2014). Estimation of Heavy metals pollution in groundwater, soil and some vegetables when treated with waste water showed concentrations above maximum limits of Fe, Cu, Zn, Mn , Ni, Cr, Cd and Pb by the above mentioned stated method (Bouksaim *et al.* ,2014). Same was revealed for municipal waste (Martin, 2014) and industrial waste (Njoku *et al*., 2013). Heavy metal toxicity detection in cabbage sections in farmlands of Ghana by random method discovered the elevated cadmium levels in inner sections while Pb and Hg were high in middle and outer sections (Larbiea *et al*., 2014).

Agricultural lands of China and their soil quality need to be handled well in order to avoid negative effects on environment. The overall impact of fertilizers and manuring on a regular basis rooted Cd, Cu and Zn in the greenhouse soils and vegetables (Zhang *et al*., 2012). In vitro studies using *Datura inoxia, Lantana camera* to examine the phytoremediation potential for heavy metals were done and conceived the idea of tissue cultured plantlets which have similar genetic characteristics and more tolerant to contaminants (Waoo *et al* . , 2014). The deposition of toxic metals in Mithi river of Mumbai is creating a harmful impact on aquatic life as well as it

is altering physical and chemical properties of water. This was tested by random method (Singare *et al*., 2012).

Composite Sampling-It does not use a definite pattern of sampling. The samples should be of equal size, and evenly spaced except geographical constraints exist. The soil samples should be taken at equal depth intervals. The extraneous materials should be removed while executing composite analysis. Nnaji (2001) showed the segmentation of heavy metals in the paddy soils of Nigeria and observed that chronic drawing of fertilizers, herbicides causes, metal build up in high levels and assessment of soil quality on regular basis is essential. Same as for application of fertilizers on rice fields and groundwater of Iran for heavy metals percentage. About 20 samples were taken before and after fertilizers application and an increase in Cadmium concentration was observed (Malidareh *et al*., 2014).

Now-a-days treated wastewater and agriculture drainage is commonly used for irrigation. Thus, quantification of heavy metals in cultivated soils showed significant presence of Cu, Pb, Ni and Zn (Mohammed *et al* ., 2014); also in Nigeria in farm soils it was seen (Agbaire *et al*., 2014).

2. Analysis of physico-chemical properties of soil

Asiriuwa *et al*., (2013) used mycoremediation technique to evaluate the bioaccumulation potential of heavy metal (Cd, Zn, Cu, Pd) by mushroom from heavy metal contaminated soils. 5 g of each sample of mushrooms were dried in the oven at 105 0C for 1 hour, 1 g of the dried sample was weighed into a 50 mL pyrex beaker and placed in a muffle furnace set at 500 0C. The mushroom was left to ash for 3 hrs. 10 mL of the ashed mushroom sample was then dissolved in 20% HNO3 and heated gently on a hot plate for about 5mins. The sample was allowed to cool and then filtered into 5 mL flask. The flask was then made to mark with water. The heavy metals in the sample were then determined using atomic absorption spectrophotometer. The physico –chemical parameters were estimated by standardized protocols. The properties estimated were pH, Cation exchange capacity by Black (1965), Particle size (Bouyoucos, 1962), Organic carbon (Nelson and Sommers, 1982), and phosphorus (Bray and Kurtyz, 1945). Estimation of Cd, Cr, Pb, pH, carbonates and CEC in soil contaminated with sewage effluents from areas of water canal of Agra and Mathura cities was done by Kumar and Nager (2012). Same was tested in Bhandra, Maharashtra by Ladwani *et al*., (2012). Ali *et al*.,

tested pH, soil texture, Total Nitrogen, P, Cu, Zn and Fe while testing AMF potential against heavy metals using wheat crop.

3. Isolation, identification, characterization and culturing of AMF

Some of the possible methods are soil trap cultures, pot substrate cultures, Plant trap cultures, root fragment cultures and multi spore cultures. In the soil trap cultures the soil of interest is accrued from site and blended with sterile soil, clay or sand. Further mixture is placed in container and seedlings or seeds are sown. The plants are maintained for establishment of Mycorrhizae and can be processed. Pot substrate cultures are modification of soil trap methods as it involves the supplementation of existing culture grown by former method to swift up the process. Another system is plant trap culture where plants are chosen and with utmost care roots are washed to wipe out any leftover traces of soil and planted in a potential substrate. Then it is observed for spore isolation which is generally a mixed culture. To produce single yield and specific cultures root fragments of selected plants are derived and communication is done with roots of mycorrhiza free hosts in a defined sterile substrate (Walker and Vestberg, 1994).

Gerdemann (1995) used spore culture method from soil or pot culture where he used a funnel string with tin foil. The neck was filled with substrate and spores were positioned at base funnel on soil. The host plant was set on funnel and allowed for mycorrhiza establishment and transferred to pot cultures. Subsistent species of *Scutellospora savannicola* were found in root samples and Rhizosphere samples amassed from Papaya plants (Khade, 2012). The root hairs were rinsed in water, dipped in 10 % KOH, acidified with 1N HCl , and stained in 0.05 % Trypton Blue in Lactoglycerol by Phillps and Hayman, 1970. Isolation of spores was performed using wet sieving and decanting method (Gerdemann and Nicolson, 1963). The identification of AMF was conveyed manually (Schenck and Perez, 1990).

AM fungi productivity is hampered when soil are irrigated with wastewater in urban area of Varanasi was perceived (Pal and Singh, 2012). Soil representatives were collected in triplicates at a depth of 0-30 cms and roots were separated, air dried, crushed passed through a 2 mm sieve, stored for estimation of soil physico-chemical properties and heavy metal concentrations in soil. The separation of AMF was done using 425 and 63 micrometers by decanting method (Gerdemann and Nicolson, 1963). After, sucrose centrifugation was done using 1.17 M sucrose

solution at a speed of 2000 rpm for 5 minutes. The supernatant was poured through 50 mm pore size mesh and cleaned with tap water. Enumeration of spores was done on Don Caster dish under dissecting microscope for morphological characteristics. *Glomus* was associated with broad host range especially with *T. erecta* and *S. bicolor*. Aggregate AMF spores slashed with increased amounts of heavy metals in soil. Though a negative correlation was associated with Nickel but slight positive effects on Cd was seen. But complete exclusion of AMF was not witnessed. Thus, phytostabilization of heavy metals by promoting plant growth to lessen the bioavailability of metals can help in purification of environment (Pal and Singh, 2012).

4. Phytoextraction of heavy metals

The protecting role of AMF for lowering the heavy metals contamination is been reported (Dueck *et al*., 1986; Dehn and Schuepp, 1989; Hildebrandt *et al*., 1999). Decrease in Pb and Mn toxicity to *Glomus intradices* by maize studied. The effect of HM toxicity was seen by measuring intraradical hyphal regrowth. Three experiments were set up; where first included the mixture of Pb and Mn in increasing concentration from 0, 0.05 and 0.1 mM including root exudates. The second set included root exudates and Pb with the same concentration and third set included Mn with same concentration. Root exudates were applied in lyophilized material per 50 $cm^3$of media. 2 minutes shaking and 5 minutes sonification was done for dissolution of exudates followed by filtration.1 mM Bis-TRIS buffer was added at pH 6.3; incubation was done in polystyrene petridishes in 0.03 cm^3 drops in dark for 5 days at 25^oC. The experiment was set up in replicates of 7. Grid line intersect method was used for measuring proliferating hyphae and mean percentage of segments was calculated. The results were analyzed using ANOVA and mean values were tested by Duncan's multiple range test (Gryndler and Malcova, 2003).

Rationale of Research

The research aims for using mycoremediation process applying Arbuscular Mycorrhizal Fungi against heavy metals from effluents of engineering and electroplating industries. The effluents in soil alter physico-chemical properties and have adverse effects on existing plant, animal and microbial communities. Extensive and continuous leaching may have altered groundwater chemical properties causing water pollution. Thus, to eliminate excess of heavy metals, AMF application can be of great advantage as it is an eco friendly, very cost effective technique. Mass production for nutrient enrichment is easy, so it is simple to manage. It can be an appropriate choice to explore and attempt to detoxify environment.

Objectives

1. Estimation of physico-chemical properties of effluents from different engineering industries.
2. To perform soil sampling (random-composite) and estimation of biological parameters of the sampled soil obtained from the industrial areas.
3. Isolation, characterization, identification and culturing of Arbuscular mycorrhizal fungi from soil and plants harboring at sites.
4. Extraction of heavy metals by application of various AMF genera *Glomus*, *Sclerocystis*, *Acaulospora*, *Enterosphospora*, *Gigaspora* and *Scutellospora* in suitable host plants Onion (*Allium cepa*) and Grass (*Cyanodon dactylon/ Cenchrus ciliaris*) from soil sample against sterile soil control and non AMF inoculated plants.
5. Integrated study with AMF, available organic amendments, Rhizobacterium and Organic manure.

Proposed Methodology

A) Collection of samples

The selected site for investigation will be sampled in a random composite manner at 0-10, 10-20, 20-30, 30-50 cms depth from different engineering industries and amounted to 2 kgs. Labeling and stored in polythene bags.

B) Estimation of Physical parameters

1. Drying of Samples

Soon after collection of samples, laying them in trays or plastic sheets shall be done. Oven drying is done for 2-4 days at a maximum of 35^{o} C and if room drying method is done then it should be done for about 7 days.

2. Sieving

Weight of air dried soil is taken and sieved through a 2 mm sieve and reweighed. The noting of proportion less than < 2 mm size is done.

3. Moisture content

10 g of air dried soil is accurately weighed. Then for overnight dried in oven at 105 ^{o} C and again reweighed.

Moisture % = Loss in weight (gms) /Weight of air dried soil (gms) *100

4. Coarse Fractions (> 2 mm size)

The coarse fraction is measured by passing the air dried samples through a 2 mm sieve and reweighing the coarse materials. It can be divided into gravel (2-75 mm diameter), cobbles (75-254 mm diameter) and stones which are greater than 254 mm in diameter.

5. Texture Analysis

To analyze the relative proportion of sand, silt and clay in a particular soil. For this, pretreatment of sample to obtain and maintain maximum dispersion of soil particles during mechanical analysis.

- Removal of Organic Matter by Hydrogen Peroxide (6%).
- Removal of carbonates by dilute HCl.
- Removal of soluble salts and carbonates by the use of Sodium Acetate.
- The dispersion of soil followed by separation of silt and clay.

6. Bulk Density and Pore Space

The soil matter contained in a unit volume of the soil sample is called bulk density. It depends on texture, structure and organic matter status of the soils. To determine the bulk density of the soil sample, the oven dried weight of a known volume of a soil sample is determined and the mass per unit volume is calculated.

6.1 Pore Space

The pore space of a soil is that portion of soil volume which is occupied by air and water.

% of pore space + % of solid space = 100%

Hence, % of pore space = 100- $\frac{\text{bulk density } (g/cm^3)}{}$ * 100

Particle density (g/cm^3)

C) Chemical analysis of soil

1. pH- The measure of H^+ ion activity of the soil. To determine whether the soil is acidic, alkaline and neutral in nature. It variability either in highly increased or decreased percentage affects plant and microbial communities. It is measured using pH meter. If the pH of soil is highly acidic then SMP buffer is preferred to neutralize the acidity.

2. Electrical Conductivity- Soil electrical conductivity (EC) is a measure of the amount of salts in soil (salinity of soil). It is an important indicator of soil health. It affects crop yields, crop suitability, plant nutrient availability, and activity of soil microorganisms. Preparation of saturated extract (ECe) by saturating about 200 to 400 gms of soil for overnight. Reweighing is

done. The saturation water percentage from the weight of oven dried soil and sum of water added is calculated.

3. Total Nitrogen

The Total nitrogen content of soil ranges from < 0.02% in subsoil's to > 2.5 % in peats. Wet oxidation procedure; the Kjeldahl method (1883) is used. It basically involves two steps:

- Digestion of sample to convert organic N to a NH^{+}_{4}-N.
- Determination of ammonium-N in the digest by distillation.

4. Available Nitrogen

To measure amount of nitrogen that may become available through microbial decomposition of the total organic nitrogen present. The procedure involves the distillation of soil sample with alkaline Potassium permanganate solution and determination of the liberated ammonia.

5. Available Phosphorus

The Brays method is widely used as an index of available P in soil. The combination of HCl and Ammonium fluoride removes acid soluble Phosphorus; Calcium phosphate and some portion of aluminum and iron phosphates. The Ammonium fluoride dissolves aluminum and iron phosphates by its complex ion formation with the metal ions.

5. Exchangeable Potassium and Sodium detection by flame photometry method.

6. Cation exchange capacity

The method is best suited for arid soils. It is determined by 1 N Ammonium acetate extract. The analysis is done using flame photometer.

7. Analysis of Ca and Mg

By Atomic absorption spectrophotometer or EDTA titrimety methods.

8. Estimation of heavy metals like Fe, Cd, Cu and by AAS.

D) Techniques for Extraction of AMF

1) Soil Sampling: Rhizosphere soil samples (Soil adhering to the roots) shall be collected at 30-90 cm depths. Then stored at 6^{o} C for further processing.

2) Spore extraction by wet sieving and decanting technique

After collection of a soil samples, suspend approximately 50 to 100 g of soil into a 2-liter container and add 1.5 liters of water. Vigorous mix the suspension to free the spores from the soil and roots. Next, heavier particles in suspension is allowed to settle for 15 to 45 (times vary depending on soil texture) and the supernatant decanted through standard sieves. Sieves should be selected so as to capture the spores of interest. A 425 μm pore size over a 45 μm pore size sieves is used for unknown fields samples. The content of the top sieve is examined for sporocarps that may be up to 1 mm in diameter. For clay soils, it is advisable to repeat the decanting and sieving procedure with the settled soil. Roots may be collected from the larger mesh sieve for evaluation of internal colonization. The sieving retained on the other different sieves was washed into separate petridishes for further observations or purification by sucrose centrifugation,

3) Sucrose Centrifugation Technique

Spores and minimal amount of organic particles could be further purified by re-suspending sieving in the 40% sucrose solution and centrifuge. Transfer sieving to 50 ml centrifuge tubes with a fine stream of water from a wash bottle and balance opposing tubes. Centrifuge at 1,200 to 1,300 x g in a swinging bucket rotor for 3 min, allowing the centrifuge to stop without braking. Remove the supernatant carefully to avoid disturbing the pellet and then with a finger remove the organic debris that adheres to the side of the tube. Suspend soil particles in chilled 1.17 M sucrose, mix the contents with a spatula and centrifuge the samples immediately at 1,200 to 1,300 x g for 1.5 min, applying the brake to stop the centrifuge. Pour the supernatant through the small mesh sieve, carefully rinse the spores held on the sieve with tap water and wash the spores into a plastic Petri dish scribed with parallel lines spaced0.5 cm a part.

4) Sterilization of AM Fungi Spore: Modified techniques of Budi et al.14 Is used to surface sterilize the spores. Spores is immersed for 10 s in 96% ethanol and washed using a 32 μm sieve. Spores are then immersed for 10 min in a solution of 0.02% streptomycin, 2% chloramines T and a drop of Twenty 20 (SCT). Subsequent washing again take place on a 32 μm sieve. A final

immersion of the spores takes place in 6% bleach for 1 min and subsequent washing in sterile distilled water. Spores are stored in sterile distilled water or water agar, or on 0.1% MgSO4 .7H2O solidified with gellan gum, in Petri dishes and glass vials in the fridge at 4°C, if not used immediately.

5) Identification by manual Schenck and Perez 1990.

6) Culturing by pot culture method.

D) Extraction of Metals by biomass estimation in terms of dry weight, total weight and grid intersect method for hyphae.

E) Statistical tools proposed to be employed

ANOVA, Correlation and regression

F) Instruments to be used

- Atomic Absorption Spectrophotometer
- Flame photometer
- Centrifuge
- Kjeldahl Distillation Assembly
- Hot air oven
- Seive-300 mm,75mm,50mm,38 mm,25 mm,10 mm,9.5mm,5.6mm,4.75mm, 0.038mm,1mm, 0.50mm,0.425mm,0.250mm,0.150mm,,.10mm,0.075mm,0.062mm,0.50mm,0.045mm.2.0mm
- pH meter
- Microscope
- Soil sampling tools Auger, spade etc

G) Sampling design

- Total sampling units for soil-15
- Host plant species- Onion and grass

H) Dependence of results on physical conditions

pH, temperature, light, Micronutrients, soil conditions

I) Reproducibility of results

The number of replicates to be taken is 6.

J) **Parameters for estimation of physico-chemical properties of effluents**

1. Color and odor
2. Suspended solids and dissolved solids
3. Temperature
4. Oil and grease
5. Biological oxygen demand
6. COD
7. Metals like As, Hg, Pb, Cd, Cr, Cu, Zn, Se, Ni, Bo etc.
8. pH
9. Acidity and alkalinity
10. Free CO_2
11. Cyanide
12. Dissolved Oxygen
13. Total hardness

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